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Structural organisation of the type IV secretion systems[☆]

Gabriel Waksman and Elena V Orlova

Type IV secretion (T4S) systems are large dynamic nanomachines that transport DNAs and/or proteins through the membranes of bacteria. Because of their complexity and multi-protein organisation, T4S systems have been extremely challenging to study structurally. However in the past five years significant milestones have been achieved by X-ray crystallography and cryo-electron microscopy. This review describes some of the more recent advances: the structures of some of the protein components of the T4S systems and the complete core complex structure that was determined using electron microscopy.

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Introduction

Nearly all types of bacteria (Gram-negative, Gram-positive, cell wall-less bacteria) and some archaea have evolved secretion systems essential for both their survival and virulence [1[•]]. Secretion systems transport DNA and effector molecules such as enzymes or toxins from the bacterial interior to its exterior. Understanding the principles of action of these nanomachines has broad clinical significance not only due to delivery of bacterial toxins or effector proteins straight into targeted host cells, but also for the direct involvement in the rapid horizontal spread of antibiotic resistance genes among the microbial population.

Secretion systems found in bacteria are classified currently into seven major classes, among which type IV secretion (T4S) systems form the most functionally versatile class [2–7]. The variety of substrates and their nature (single proteins, protein complexes, DNA and nucleoprotein complexes) secreted by the T4S systems indeed single out this class of secretion systems from the others. The T4S systems are classified into three functional groups. The first group mediates the transfer of DNA from one bacterial cell to another in a process called conjugation that plays an important role in bacterial genome plasticity and diversity. Another group of T4S systems mediates the translocation of proteins, ranging from small protein effectors to large protein complexes. Pathogenic Gram-negative bacteria such as *Helicobacter pylori*, *Brucella suis* and *Legionella pneumophila* use the T4S system to inject virulence proteins into mammalian host cells [8–10] and *Bordetella pertussis* use the T4S systems to secrete pertussis toxin into the extracellular milieu [11]. The third group mediates DNA release and uptake. *H. pylori* and *Neisseria gonorrhoeae* typify bacteria with this type of T4S systems [13]. T4S systems of the first class represent an enormous public-health problem as they are involved in the rapid dissemination of antibiotic-resistance genes and other virulence traits among pathogens. While the fact that DNA can move from one cell to another has been established a long time ago [12,15], the mechanism of secretion was poorly understood since no structural information was available until very recently.

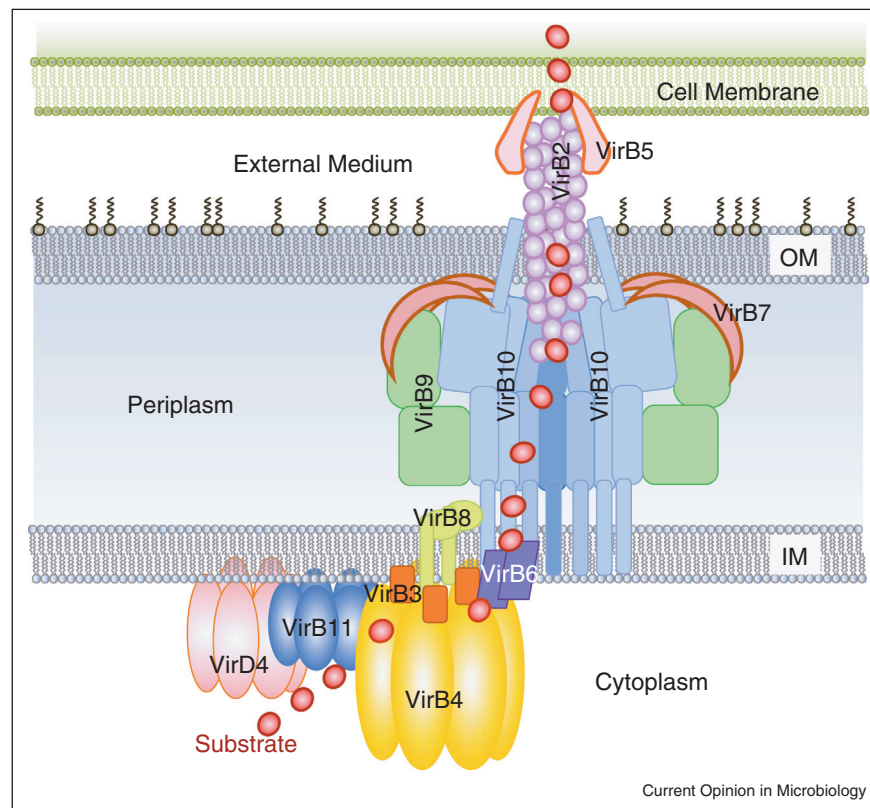
The T4S systems are evolutionarily related: all nucleoprotein and protein complexes are secreted by the T4S systems in an ATP-dependent process using a specific channel through a cell envelope [14]. The T4S systems share a number of main components and it seems that the secretion process of all types of substrates has common features [16]. In this review we describe the latest progress in structural studies of the T4S system components and their major complexes.

Overall organisation of T4S systems

It has been shown that the T4S systems span the entire bacterial cell envelope, creating a translocation channel for various substrates [1[•]]. Almost all T4S systems of Gram-negative bacteria minimally consist of 12 proteins named VirB1 to VirB11 and VirD4 (based on the nomenclature used for *Agrobacterium tumefaciens* T4S system) that form a multi-protein envelope-spanning transport apparatus (Figure 1). Each protein is present in multiple copies. The twelve components are organised in three major subcomplexes. A cytoplasmic-inner membrane (IM) subcomplex is composed of three ATPases (VirB4,

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Figure 1



Overall organisation of the T4S system. VirD4 (in pink), VirB11 (in blue), VirB4 (in gold) ATPases, polytopic VirB6 (in purple), bitopic VirB8 (in light green) and VirB3 (in orange) form the cytoplasmic IM part of the complex. VirB7 (in brown), VirB9 (in green), and VirB10 (in blue) compose the periplasmic part of the secretion system. VirB2 and VirB5 constitute the outer part of the secretion system. Red dot indicates the path of the substrate through the machinery as established by Cascales and Christie [22]. The stoichiometry of the various components in a native, fully assembled, T4S system is unknown.

VirB11 and VirD4) and the VirB3, VirB6 and parts of the VirB8 and VirB10 proteins. The ATPases power system assembly and substrate translocation [17–21]. VirB3, VirB6, and VirB8 are likely to anchor VirB4 (and also perhaps VirB11) to the IM, participate in the IM channel, and provide a link to the core complex (CC). The CC is composed of three proteins: VirB7, VirB9 and VirB10, and is a large central structure of the T4S system, that serves as scaffolding for the rest of the T4S system components. This complex forms a trans membrane spanning pore inserted both in the outer membrane (OM) and IM of Gram-negative bacteria and participates actively in T4S substrate transfer through the bacterial envelope [1, 22, 23, 24]. The third subcomplex is formed by the VirB2 and VirB5 proteins that make the extracellular pilus that is important for direct contact with the recipient cell surface and may act as a conduit delivering the substrate to the recipient cell (Figure 1). While there are some deviations in the composition of these subcomplexes between different types of T4S systems they are likely conserved in their overall organisation [1].

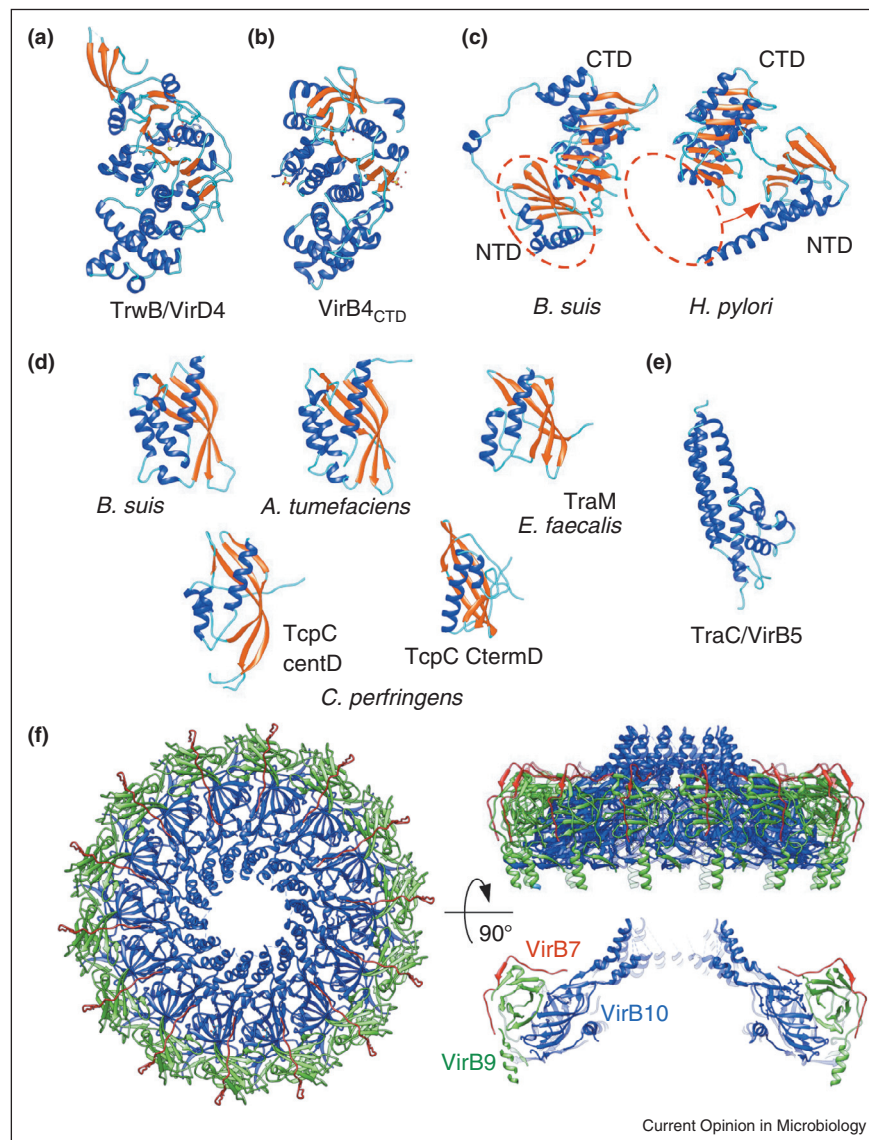
Advances in the structural analysis of T4S systems

The complexity and dynamics of the T4S systems and their multiprotein organisation represent serious difficulties for the structural analysis of T4S systems or even their components. Only during the last decade structural information has emerged for the VirB/D system from *A. tumefaciens* and the closely related systems from *Escherichia coli* encoded by the conjugative plasmids F, R388, pKM101 and RP4 [25, 26]. X-ray crystallographic studies of the T4S system have been performed on soluble protein components (VirB11, the cytoplasmic regions of VirD4 and VirB4, the periplasmic domain of VirB8, and VirB5) and on the OM-embedded region of the CC (Figure 2).

Structures of ATPases

VirD4 homologues are essential T4S ATPases that act as substrate receptors at the entry of the T4S apparatus and might power substrate translocation through the IM [21]. Because of their role in delivering the substrate to the translocation channel, VirD4 proteins are termed

Figure 2



Structures of T4S system components or domains. (a) VirD4: structure of the soluble domain of TrwB. One subunit of the hexamer is shown (coils are shown in cyan, helices in blue, and strands in red). (b) VirB4: C-terminal domain of *Thermoanaerobacter pseudethanolicus* VirB4; (c) VirB11: crystal structures of *B. suis* VirB11 and *H. pylori* HP5025, CTD-C terminal domain, NTD-N terminal domain (shown in red dashed oval on *B. suis* VirB11). Red arrow indicates the shift of the NTD in *H. pylori* Vir11 compared to *B. suis* VirB11. (d) VirB8: crystal structure of the periplasmic domain (C-terminal domain) of VirB8 from *B. suis* (left panel), *A. tumefaciens* (middle panel), TraM₂₁₄₋₃₂₂ protein (right panel); the central and C-terminal domains of the TcpC₉₉₋₃₅₉ structure are shown in the bottom panel. (e) VirB5: crystal structure of TraC encoded by the *E. coli* conjugative plasmid pKM101. (f) Crystal structure of the CC's O-layer composed of VirB7 (in dark red), VirB9_{CT} (in green) and VirB10_{CT} (blue). All structures are shown in the ribbon representation.

'coupling proteins'. The crystal structure of the soluble ~50 kDa cytoplasmic domain of TrwB (VirD4 homologue) from the R388 conjugative plasmid (PDB 1GKI, Figure 2A) revealed a homohexamer in which each subunit has two distinct domains: an all-alpha domain facing the cytoplasm and an ATP-binding domain linked to the IM by the N-terminal membrane anchor [17]. The structure implies that VirD4 could function as a rotary motor that assists ssDNA and/or protein translocation through

the IM. Another recent evidence of involvement of coupling proteins in engagement of substrates and activation of the secretion system was demonstrated for DotL, an IM component of the Dot/Icm secretion system in *L. pneumophila*. DotL acts as an IM receptor for substrates and requires adaptor proteins for the secretion of a major class of substrates [27]. The DotL protein is analogous to VirD4 in *A. tumefaciens*, and TraB in *E. coli* pKM101 plasmid [28]. As in all members of the VirD4

family, DotL also contains a Walker A motif for ATP hydrolysis and is believed to provide energy to actively drive the export of substrates across the inner and outer membranes of the bacterial cell wall [27].

The VirB4 ATPase of the T4S system is also essential for the assembly of the system and substrate transfer. Recently the crystal structure of the C-terminal domain of *Thermoanaerobacter pseudethanolicus* VirB4 (PDBs 4AG6 and 4AG5) has been obtained [20]. This structure is similar to VirD4, although it shares only 12% sequence identity. The VirB4 domain crystallizes as a monomer, but the full-length protein is observed in monomer–dimer equilibrium, even in the presence of nucleotides and/or DNAs. The VirB4 proteins share four common sequence motifs including the consensus Walker A and B nucleoside triphosphate-binding motifs (Figure 2B). A VirB4 topology model suggests possible periplasmic loops, one near the N terminus and a second on the N-terminal side of the Walker A motif [20]. The cellular localization, topology and oligomerization state of VirB4 remain unclear: different studies have reported dimeric and higher oligomeric (tetrameric and hexameric) forms of the protein [29–32]. It was shown that VirB4 can be bound through its N-terminal domain to the core's VirB9 protein [20]. VirB4 is involved in earlier steps of substrate engagement, and makes a number of additional interactions, notably with VirB3 and VirB8 [33–35].

VirB11 is a peripheral membrane protein and belongs to a family of ATPases termed 'traffic ATPases' [19,18,36]. Recent studies have shown that conformation of VirB11(TrwD) can be regulated by magnesium, that at physiological concentrations induces a more rigid conformation of the enzyme hence slowing down its activity [37]. The crystal structure of the ADP-bound *H. pylori* VirB11 homologue (PDB 2PT7) revealed that each monomer consists of two domains formed by the N-terminal and C-terminal halves of the protein [19]. The nucleotide-binding site is at the interface between the two domains. In the hexamer, the N-terminal domain and C-terminal domain form two separate rings defining a chamber of ~50 Å. Comparison of the structures of *B. suis* (2GZA) and *H. pylori* VirB11 (PDB 2PT7) showed that the *B. suis* VirB11 monomer differs significantly from that of *H. pylori* by a N-terminal domain swap that greatly modifies the nucleotide-binding site and the interface between subunits (Figure 2C) [18].

Structures of VirB5 and VirB8 proteins

VirB8 homologues are essential for substrate transfer through the IM. It has been proposed that they are part of the inner-membrane pore and interact with VirB3, VirB4, VirB5 and VirB6 [35,38,39]. The N-terminal extremity of the structure is connected to a transmembrane segment inserted in the IM. A structure of the periplasmic domain (C-terminal domain) of VirB8 has been obtained

for *B. suis* and *A. tumefaciens* (PDBs 2BHM and 2CC3) [38–40]. Structural comparison of *B. suis* and *A. tumefaciens* VirB8 confirms that the monomers have a similar fold (Figure 2D). It comprises an extended β -sheet flanked with α -helices. Both proteins exist as homodimers, and amino acids at the dimer interface are critical for protein function. Smith *et al.* have found that E115 and K182 interact with inhibitors which apparently induce conformational changes that prevents VirB8–VirB8 and VirB8–VirB10 interactions [41^{*}]. Recently it was shown that the C-terminal component of the transfer TraM protein (214–322 aa, PDB 4EC6) from the *Enterococcus faecalis* conjugative plasmid pIP501 and the conjugation protein TcpC_{99–359} (PDB 3UB1) from *Clostridium perfringens* (Gram-positive) plasmid pCW3 are structurally homologous to the periplasmic region of VirB8 [42^{**},43^{**}]. TraM and TcpC are found localized to the cell membrane. Despite the absence of sequence-based similarity, the crystal structures of both proteins display folds similar to the T4S system VirB8 proteins from *B. suis* and *A. tumefaciens*. Surprisingly, the TcpC_{99–359} structure comprises two VirB8-like domains separated by a linker. The C-terminal domain is critical for interactions with other conjugation proteins (Figure 2D). The structure, molecular dynamics, and cross-linking studies indicate that TraM is active as a trimer. The oligomerization state of TcpC is not known.

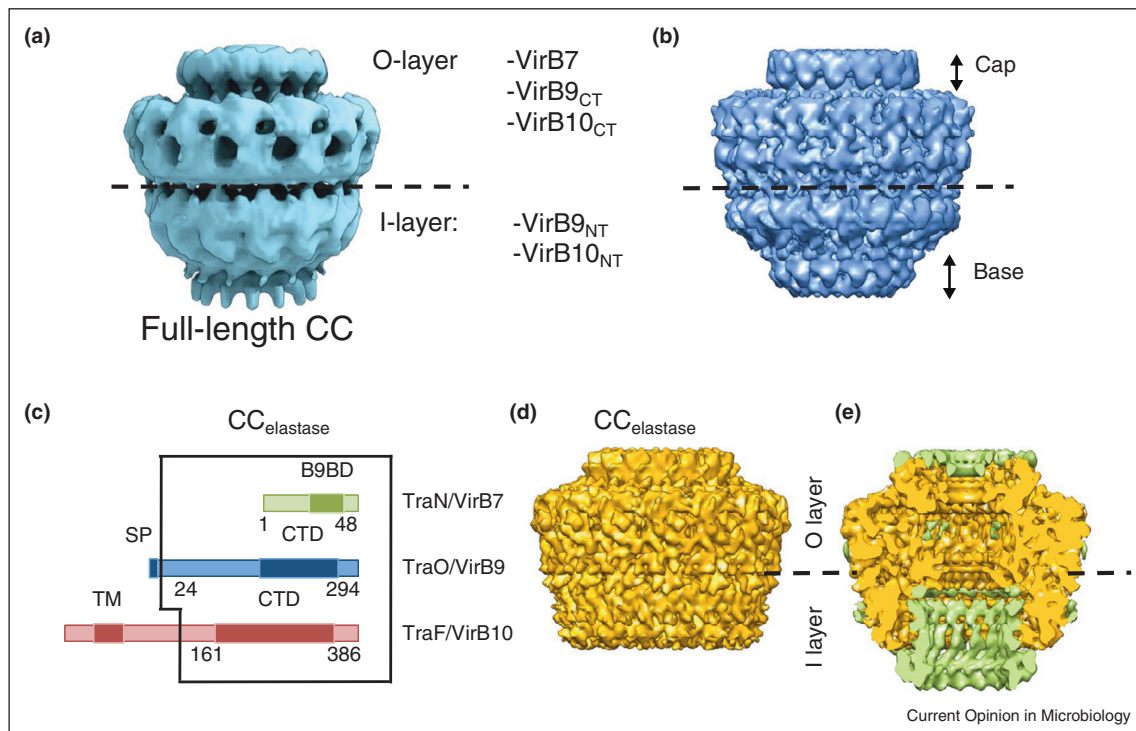
The crystal structure of TraC, a VirB5 homologue encoded by the *E. coli* conjugative plasmid pKM101 (PDB 1R8I, Figure 2E), has been obtained at 3 Å resolution [44]. The VirB5 structure comprises a 3-helix bundle flanked by a smaller globular domain. Together with the main pilin VirB2, VirB5 homologues form the T4S pilus, possibly acting as adhesins.

Structures of the CCs

The most advanced structural work described to date is the X-ray crystal structure for the part of the CC that locates in and near the OM. This part, termed the 'O-layer', forms the OM pore of the T4S system (PDB 3JQO) [45^{**}]. It is composed of three proteins/domains: the TraF/VirB10 C-terminal domain (TraF/VirB10_{CT}), the TraO/VirB9 C-terminal domain (TraO/VirB9_{CT}), and TraN/VirB7, each in 14 copies. The structure has unveiled a surprisingly intricate network of interactions between these three major components of the CC (Figure 2F). VirB7 seems to work as a stapler that fastens VirB9 and VirB10 together; the inner surface of the OM channel is lined by VirB10. A diameter of the α -helical pore in the crystal structure is ~32 Å that could accommodate the passage of DNA and unfolded protein substrates.

Experiments combining cryo-EM structure determination of full-length CC and partly deleted CCs, docking of X-ray crystallographic and pseudo atomic models, and immuno-labelling have resulted in the elucidation of the

Figure 3



Electron microscopy of the T4S system. (a) Structure of the CC complex at 15 Å; (b) structure of the CC complex at 12 Å; (c) schematic illustration of the regions of TraN/VirB7, TraO/VirB9, and TraF/VirB10 present in the CC_{elastase} complex. Domains corresponding to the VirB9 binding domain (B9BD), the signal peptide (SP), the N-terminal trans-membrane (TM) helix and the C-terminal domains (CTD) are shown in darker colours; (d) Cryo-EM structure of the CC_{elastase} complex. (e) Cutaway view of the superposition of the difference map (in green) between the full length CC and CC_{elastase} and the cryo-EM structure of the CC_{elastase} complex (in gold).

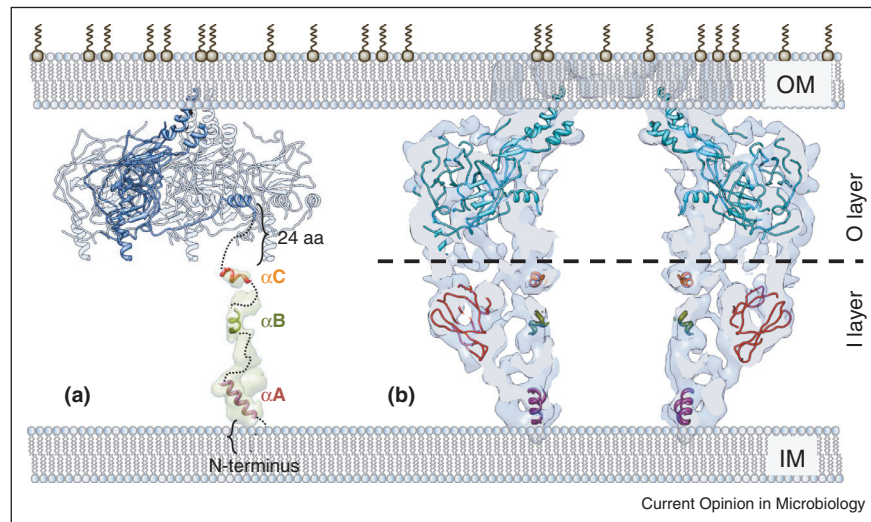
structure of the entire CC (Figure 3). The first structure of the complete CC (Figure 3A) was solved by cryo-electron microscopy (cryo-EM) at 15 Å resolution using images of frozen samples [25]. The overall dimensions of the complex are 185 Å in both height and diameter. It consists of two layers, termed O and I. The O-layer has an inner chamber that is open to the extracellular media through an opening of 10–20 Å in the cap. The complex has an inner channel that spans the structure from the OM to the IM. Later on, the CC structure was refined to a resolution of 12.4 Å (Figure 3B) which revealed more details of the inner central channel [46]. In this higher resolution structure, the I-layer has a diameter of ~50–55 Å and its inner wall is formed by 14 columns of density with a diameter of ~8 Å. The columns project from the O-layer of the CC to the bottom of the I-layer where they are connected to the base of the complex defining an opening of ~55 Å in diameter on the cytoplasmic side of the complex.

Digestion of the CC with elastase produced a stable truncated complex, termed the 'CC_{elastase}' complex. The sequencing of the bands in SDS-PAGE gels confirms that the complex is made of full-length TraN/VirB7 and

TraO/VirB9, and TraF/VirB10_{CT} (Figure 3C). A structure of CC_{elastase} was obtained at a resolution of 8.5 Å using cryo-EM and single particle analysis (Figure 3D) [46]. Comparison of full length CC and CC_{elastase} demonstrated considerable differences in the I-layer region. In the full-length CC, the I-layer comprises the N-terminal domains of VirB9 (TraF/VirB9_{NT}) and VirB10 (TraF/VirB10_{NT}) and is inserted in the IM via the VirB10 N-terminal trans-membrane segment. The removal of TraF/VirB10_{NT} by elastase results in an increase of the opening on the cytoplasmic side (65 Å compared to ~55 Å in the CC) and a shortening of the I-layer (Figure 3D).

In the CC_{elastase} complex, the columns within the central channel are absent (Figure 3E). Therefore the inner columns of the CC I-layer were assigned to TraF/VirB10_{NT} (Figure 4A). The major part of TraF/VirB10_{NT} was shown to be unstructured; the only secondary structural elements that were reliably identified were three short helical elements: the helix corresponding to the inner-membrane spanning region (αA: K₄₀AF-LVF₅₃) and two other α-helical regions (αB: A₁₀₇RA-QAA₁₁₃; αC: P₁₃₈EE-QRR₁₄₆). Their length is compatible with the diameter and the length of the observed columns in

Figure 4



Schematic model for the full length organisation of the core complex. (a) Four TraF/VirB10_{CT} subunits of the 14-mer present in the O-layer atomic structure are shown. One subunit is highlighted in blue. The density of one subunit column in the difference map (in light green) is shown with the tentative docking of the three TraF/VirB10_{NT} α-helical regions. This subunit is located immediately below TraF/VirB10_{CT} shown in dark blue. The connections are shown in dashed lines. (b) Central slice of the full length CC with fitted O-layer atomic structure (in cyan) and atomic models obtained for TraO/VirB9_{NT} (in dark red) and the α-helices predicted in TraF/VirB10_{NT} (shown as in (a)).

the CC cryo-EM map ([46^{••}], and references herein)]. At the very bottom of the complex these stretches join each other to form a ring at the base of the CC. This ring might correspond to the 14 trans-membrane segments (helix αA) associated with detergent micelles. VirB10 is linked to the IM by the VirB10 N-terminal trans-membrane helix (30–50 aa) characterised with two types of putative dimerization motifs: a GxxxA (GA4) motif and two leucine (Leu1, Leu2) zippers. While mutations in the Leu1 motif disrupt T-pilus biogenesis, this and other mutations in the GA4 or Leu2 motif do not abolish substrate transfer [47].

The modelling of the atomic structure of TraO/VirB9_{NT} (residues 24–135) and the examination of its possible docking into EM maps helped locate this domain within the structure. The results suggest that TraO/VirB9_{NT} adopts a beta-sandwich fold (Figure 4B). Fitting of models for TraO/VirB9_{NT}, and labelling experiments with nanobodies targeted against TraO/VirB9_{NT} are all pointing towards the conclusion that TraO/VirB9_{NT} is located at the outside surface of the I-layer.

Conclusions

During the last decade structural analysis has made a great impact on our understanding of how T4S systems are organised and function.

The EM structures have greatly altered our conception of the entire T4S system architecture and its possible mechanism of action. It was found that the CC is an

essential structural portion of T4S systems. It is self-assembling and therefore is likely to be assembled first. The CC forms the IM channel and is a major component of the OM pore. It provides the central scaffold for assembly of all other components. Structural comparison of the complete and truncated CCs demonstrate that TraF/VirB10 spans the entire cell envelope. This observation together with fitting of crystal and modelled structures into EM maps of large complexes is of particular interest since it provides the explanation how VirB10 is able to sense conformational changes induced by ATP binding to or ATP hydrolysis by the T4S system ATPases situated at the T4S channel's entrance on its cytoplasmic side [23]. VirB10 responds to these changes by undergoing its own structural modifications that are required for substrate transfer. Cascales *et al.* have demonstrated that DNA binding to VirD4 and VirB11 stimulates ATP binding/hydrolysis, which in turn activates VirB10 through a structural transition [48[•]]. Segure and co-authors have shown that the transmembrane domain of TrwB/VirD4 is needed for the interaction with the transmembrane domain of TrwB/VirB10 and for R388 conjugative transfer. The removal of first 64 amino acids in TrwB/VirB10 reduced conjugation by six orders of magnitude [47]. Thus proteins analogues to VirB10 may serve as a signal transmitter throughout the secretion complex [1[•],21,23,25[•],47,48[•],49[•]].

The next mission in T4S studies is to establish the organisation and structure of even larger complexes of T4S system components. For example, it is important to

complete our understanding of the IM channel by determining the structure of VirB6 and VirB8 bound to the CC. Ultimately, the structure of the entire translocation machine will need to be determined. A related question is how substrate transfer takes place. This task represents a tremendous challenge that can only be met by implementing novel biochemical and genetic approaches resulting in trapping T4S systems at different stages of substrate transfer. The elucidation of T4S mechanisms will thus require joint efforts from biochemistry and structural biology. The derived knowledge might be used to find ways of inhibiting these systems to combat infections and control the spread of antibiotic resistance genes.

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